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Inventors: **Hellstrand, Kristoffer; Hermodsson, Svante; Gehlsen, Kurt R.**

For: **ACTIVATION AND PROTECTION OF CYTOTOXIC LYMPHOCYTES USING A REACTIVE OXYGEN METABOLITE INHIBITOR**

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ACTIVATION AND PROTECTION OF CYTOTOXIC LYMPHOCYTES USING A REACTIVE OXYGEN METABOLITE INHIBITOR

Related Applications

This application claims priority from U.S. Provisional Patent Application No. 60/144,394, filed on July 16, 1999.

Field of the Invention

The invention disclosed herein relates to methods of treating cancer or viral diseases in which a reactive oxygen metabolite (ROM) inhibitor is administered alone or in conjunction with additional agents. The administration of these various agents results in the activation and protection of cytotoxic lymphocytes from the deleterious and inhibitory effects of monocytes/macrophages (MO), as well as a stimulation of the anti-cancer and anti-viral properties of cytotoxic lymphocytes. In addition, antigen presenting cells may become more effective at antigen presentation to certain cytotoxic lymphocytes as a direct effect of ROM inhibitor administration. The addition of other agents that are cytotoxic lymphocyte activation compounds that stimulate the cytotoxic activity of these lymphocytes, preferably in a synergistic fashion with a ROM inhibitor are also contemplated. Representatives of such immunological stimulatory compounds include cytokines, peptides, flavonoids, vaccines, and vaccine adjuvants. Additional classes of agents usable with the methods of the invention encompass chemotherapeutic and/or antiviral agents. The invention also contemplates the use of reactive oxygen metabolite scavengers in conjunction with the above mentioned compounds.

Background of the Invention

The immune system has evolved complex mechanisms for recognizing and destroying foreign cells or organisms present in the body of the host. Harnessing the body's immune mechanisms is an attractive approach to achieving effective treatment of malignancies and viral infections.

The immune system has two types of responses to foreign bodies based on the components which mediate the response: a humoral response and a cell-mediated

response. The humoral response is mediated by antibodies while the cell-mediated response involves cells classified as lymphocytes. Recent anticancer and antiviral strategies have focused on utilizing the cell-mediated host immune system as a means of anticancer or antiviral treatment or therapy. A brief review of the immune system will assist in placing the invention in context.

Generation of an Immune Response

The immune system functions in three phases to protect the host from foreign bodies: the cognitive phase, the activation phase, and the effector phase. In the cognitive phase, the immune system recognizes and signals the presence of a foreign antigen or invader in the body. The foreign antigen can be, for example, a cell surface marker from a neoplastic cell or a viral protein. Once the system is aware of an invading body, the cells of the immune system proliferate and differentiate in response to the invader-triggered signals. The last stage is the effector stage in which the effector cells of the immune system respond to and neutralize the detected invader.

A wide array of effector cells implement an immune response to an invader. One type of effector cell, the B cell, generates antibodies targeted against foreign antigens encountered by the host. In combination with the complement system, antibodies direct the destruction of cells or organisms bearing the targeted antigen.

Another type of effector cell is the cytotoxic lymphocyte. The natural killer cell (NK cell), a type of cytotoxic lymphocyte having the capacity to spontaneously recognize and destroy a variety of virus infected cells as well as malignant cell types. The method used by NK cells to recognize target cells is poorly understood.

Another type of cytotoxic lymphocyte is the T-cell. T-cells are divided into three subcategories, each playing a different role in the immune response. Helper T-cells secrete cytokines which stimulate the proliferation of other cells necessary for mounting an effective immune response, while suppressor T-cells down regulate the immune response. A third category of T-cell, the cytotoxic T-cell (CTL), is capable of directly lysing a targeted cell presenting a foreign antigen on its surface.

The Major Histocompatability Complex and T Cell Target Recognition

T-cells are antigen specific immune cells, that function in response to specific antigen signals. B lymphocytes and the antibodies they produce are also antigen specific entities. However, unlike B lymphocytes, T-cells do not respond to antigens in a free or soluble form. For a T-cell to respond to an antigen, it requires the antigen to be bound to a presenting complex known as the major histocompatibility complex (MHC).

MHC complex proteins provide the means by which T-cells differentiate native or "self" cells from foreign cells. There are two types of MHC, class I MHC and class II MHC. T Helper cells ($CD4^+$) predominately interact with class II MHC proteins while cytolytic T-cells ($CD8^+$) predominately interact with class I MHC proteins. Both MHC complexes are transmembrane proteins with a majority of their structure on the external surface of the cell. Additionally, both classes of MHC have a peptide binding cleft on their external portions. It is in this cleft that small fragments of proteins, native or foreign, are bound and presented to the extracellular environment.

Cells called antigen presenting cells (APCs) display antigens to T-cells using the MHC complexes. For T-cells to recognize an antigen, it must be presented on the MHC complex for recognition. This requirement is called MHC restriction and it is the mechanism by which T-cells differentiate "self" from "non-self" cells. If an antigen is not displayed by a recognizable MHC complex, the T-cell will not recognize and act on the antigen signal.

T-cells specific for the peptide bound to a recognizable MHC complex bind to these MHC-peptide complexes and proceed to the next stage of the immune response.

Cytokines Involved In Mediating the Immune Response

The interplay between the various effector cells listed above is influenced by the activities of a wide variety of chemical factors which serve to enhance or reduce the immune response as needed. Such chemical modulators may be produced by the effector cells themselves and may influence the activity of immune cells of the same or different type as the factor producing cell.

One category of chemical mediators of the immune response is cytokines, molecules which stimulate a proliferative response in the cellular components of the immune system.

Interleukin-2 (IL-2) is a cytokine synthesized by T-cells which was first identified in conjunction with its role in the expansion of T-cells in response to an antigen (Smith, K.A. Science 240:1169 (1988)). It is well known that IL-2 secretion is necessary for the full development of cytotoxic effector T-cells (CTLs), which play an important role in the host defense against viruses. Several studies have also demonstrated that IL-2 has antitumor effects that make it an attractive agent for treating malignancies (see e.g. Lotze, M.T. et al, in "Interleukin 2", ed. K.A. Smith, Academic Press, Inc., San Diego, CA, p237 (1988); Rosenberg, S., Ann. Surgery 208:121 (1988)). In fact, IL-2 has been utilized to treat subjects suffering from malignant melanoma, renal cell carcinoma, and acute myelogenous leukemia. (Rosenberg, S.A., *et al.*, N. Eng. J. Med. 316:889-897 (1978); Bukowski, R. M., *et al.*, J. Clin. Oncol 7:477-485 (1989); Foa, R., *et al.*, Br. J. Haematol. 77:491-496 (1990)).

Another cytokine with promise as an anticancer and antiviral agent is interferon-α. Interferon-α (IFN-α) is an IFN type I cytokine, has been employed to treat leukemia, myeloma, and renal cell carcinomas. IFN type I cytokines have been shown to increases class I MHC molecule expression. Because most cytolytic T-cells (CTLs) recognize foreign antigens bound to class I MHC molecules, type I IFNs may boost the effector phase of cell-mediated immune responses by enhancing the efficiency of CTL-mediated killing. At the same time, type I IFN may inhibit the cognitive phase of immune responses, by preventing the activation of class II MHC-restricted helper T-cells. IL-12, IL-15, and various flavonoids can also increase the T-cell response.

In vivo results of histamine agonist treatments

Histamine is a biogenic amine, i.e. an amino acid that possesses biological activity mediated by pharmacological receptors after decarboxylation. The role of histamine in immediate type hypersensitivity is well established. (Plaut, M. and Lichtenstein, L.M. 1982 Histamine and immune responses. In Pharmacology of Histamine Receptors, Ganellin, C.R. and M.E. Parsons eds. John Wright & Sons, Bristol pp. 392-435.)

Examinations of whether a H_2-receptor agonists or antagonists can be applied to the treatment of cancer have yielded contradictory results. Some reports suggest that administration of histamine alone suppressed tumor growth in hosts having a malignancy. (Burtin, Cancer Lett. 12:195 (1981)). On the other hand, histamine has been reported to accelerate tumor growth in rodents. (Nordlund, J.J., *et al.*, J. Invest. Dermatol 81:28 (1983)).

Similarly, contradictory results were obtained when the effects of histamine-receptor antagonists were evaluated. Some studies report that histamine-receptor antagonists suppress tumor development in rodents and humans. (Osband, M.E., *et al.*, Lancet 1 (8221):636 (1981)). Other studies report that such treatment enhances tumor growth and may even induce tumors. (Barna, B.P., *et al.*, Oncology 40:43 (1983)).

<u>Synergistic Effects of a H_2-receptor agonist and IL-2</u>

Despite the conflicting results when histamine is administered alone, recent reports clearly reveal that histamine acts synergistically with cytokines to augment the cytotoxicity of NK cells. For example, studies using histamine analogues suggest that histamine's synergistic effects are exerted through the H_2-receptors expressed on the cell surface of monocytes. (Hellstrand, K., *et al.*, J. Immunol. 137:656 (1986)).

Histamine's synergistic effect when combined with cytokines appears to result from the suppression of a down regulation of cytotoxicity mediated by other cell types present along with the cytotoxic cells. *In vitro* studies with NK cells alone confirm that cytotoxicity is stimulated when IL-2 is administered. However, in the presence of monocytes, the IL-2 induced enhancement of cytotoxicity of NK cells is suppressed. (See, U.S. Patent Number 5,348,739, which is incorporated herein by reference).

In the absence of monocytes, histamine had no effect or weakly suppressed NK mediated cytotoxicity. (Hellstrand, K., *et al.*, J. Immunol. 137:656 (1986); Hellstrand, K. and Hermodsson, S., Int. Arch. Allergy Appl. Immunol. 92:379-389 (1990)). Yet, NK cells exposed to histamine and IL-2 in the presence of monocytes exhibit elevated levels of cytotoxicity relative to that obtained when NK cells are exposed only to IL-2 in the presence of monocytes. *Id.* Thus, the synergistic enhancement of NK cell cytotoxicity by combined histamine and interleukin-2 treatment results not from the direct action of

histamine on NK cells but rather from suppression of an inhibitory signal generated by monocytes.

Without being limited to a particular mechanism, it is believed that the inhibitory effects of monocytes on NK-cell cytotoxicity result from the generation of reactive oxygen metabolites such as H_2O_2 by monocytes. Hydrogen peroxide may be generated within the cell. Alternatively, H_2O_2 may be catalyzed by enzymes located on the surface of MO cells. Both sources of H_2O_2 are thought to contribute to intercellular H_2O_2 concentrations.

Granulocyte have also been shown to suppress IL-2 induced NK-cell cytotoxicity *in vitro*. It appears that the H_2-receptor is involved in transducing histamine's synergistic effects on overcoming granulocyte mediated suppression. For example, the effect of histamine on granulocyte mediated suppression of antibody dependent cytotoxicity of NK cells was blocked by the H_2-receptor antagonist ranitidine and mimicked by the H_2-receptor agonist dimaprit. In contrast to the complete or nearly complete abrogation of monocyte mediated NK cell suppression by histamine and IL-2, such treatment only partially removed granulocyte mediated NK cell suppression. (U.S. Patent Number 5,348,739; Hellstrand, K., *et al.*, Histaminergic regulation of antibody dependent cellular cytotoxicity of granulocytes, monocytes and natural killer cells., J. Leukoc. Biol 55:392-397 (1994)).

As suggested by the experiments above, therapies employing histamine and cytokines are effective anticancer and antiviral strategies. U.S. Patent Number 5,348,739 discloses that mice given histamine and IL-2 prior to inoculation with melanoma cell lines were protected against the development of lung metastatic foci. It has also been shown that a single dose of histamine could prolong survival time in animals inoculated intravenously with herpes simplex virus (HSV), and a synergistic effect on the survival time of animals treated with a combination of histamine and IL-2 was observed (Hellstrand, K., *et al.*, Role of histamine in natural killer cell-dependent protection against herpes simplex virus type 2 infection in mice., Clin. Diagn. Lab. Immunol. 2:277-280 (1995)).

The above results demonstrate that strategies employing a combination of histamine and IL-2 are an effective means of treating malignancies and viral infection.

Presently the therapeutic potential of several immune cell stimulating compounds that show promise as efficacious anticancer and antiviral agents is diminished due to negatively regulating systems of the immune system. Accordingly, there is a need for methods which maximize the therapeutic potential of immune cell stimulating compounds.

Summary of the Invention

The invention relates to methods and compositions for facilitating activation and protection of cytotoxic lymphocytes. In one embodiment the invention relates to a method comprising identifying a patient in need of enhanced cytotoxic lymphocyte activity, and administering to the patient an amount of diphenylionodonium (DPI) effective to activate and protect cytotoxic lymphocyte function in the presence of MO.

In another embodiment, the method further comprises administering an a cytotoxic lymphocyte stimulatory composition. In various aspects of this embodiment, the composition may be a vaccine adjuvant, a vaccine, a peptide, a cytokine or a flavonoid. Vaccine adjuvants for use with the invention may be selected from the group consisting of bacillus Calmette-Guerin (BCG), pertussis toxin (PT), cholera toxin (CT), *E. coli* heat-labile toxin (LT), mycobacterial 71-kDa cell wall associated protein, microemulsion MF59, microparticles of poly(lactide-co-glycolides)(PLG), and immune stimulating complexes (ISCOMS). Vaccines for use with the invention may be selected from the group consisting of influenza vaccines, human immunodeficiency virus vaccines, *Salmonella enteritidis* vaccines, hepatitis B vaccines, *Boretella bronchiseptica* vaccines, tuberculosis vaccines, allogeneic cancer vaccines, and autologous cancer vaccines. The invention contemplates the use of a variety of cytokines and flavonoids. The cytokines may be selected from IL-1, IL-2, IL-12, IL-15, IFN-α, IFN-β, or IFN-γ. Flavonoids may be selected from the group consisting of flavone acetic acids and xanthenone-4-acetic acids. These compounds may be administered in a daily dose to an adult human of between 1000 and 600,000 U/kg.

Another embodiment of the invention contemplates the use of compounds effective to inhibit the production or release of intercellular hydrogen peroxide selected from the group consisting of histamine, histamine phosphate, serotonin, dimaprit,

clonidine, tolazoline, impromadine, 4-methylhistamine, betazole, and a histamine congener. In one aspect of this embodiment, these compounds may be administered to an adult human at between 0.05 and 50 mg per dose. In another aspect of this embodiment, these compounds may also be administered at between 1 and 500 μg/kg of patient weight per dose.

Another embodiment of the invention contemplates administration of the cytotoxic lymphocyte activating compound and the ROM inhibitory compound within 1 hour of each other. Another embodiment contemplates administration of the cytotoxic lymphocyte activating and protecting compound and the ROM inhibitory compound within 24 hours of each other.

The methods of the invention further contemplate an embodiment in which an effective amount of a scavenger of intercellular hydrogen peroxide is administered. In one aspect of this embodiment, the scavenger may be selected from the group consisting of catalase, glutathione peroxidase, and ascorbate peroxidase. In another aspect of this embodiment, the hydrogen peroxide scavenger may be administered to an adult human in a dose of from about 0.05 to about 50 mg/day and the compounds maybe administered together or separately.

In addition to the compounds discussed above, the invention contemplates the administration of a variety of chemotherapeutic agents. In one embodiment, the chemotherapeutic agent is an anticancer agent selected from the group consisting of cyclophosphamide, chlorambucil, melphalan, estramustine, iphosphamide, prednimustin, busulphan, tiottepa, carmustin, lomustine, methotrexate, azathioprine, mercaptopurine, thioguanine, cytarabine, fluorouracil, vinblastine, vincristine, vindesine, etoposide, teniposide, dactinomucin, doxorubin, epirubicine, bleomycin, nitomycin, cisplatin, carboplatin, procarbazine, amacrine, mitoxantron, tamoxifen, nilutamid, and aminoglutemide. Conventional dosages of these agents can be used. In another embodiment, the chemotherapeutic agent administered is an antiviral agent, selected from the group consisting of idoxuridine, trifluorothymidine, adenine arabinoside, acycloguanosine, bromovinyldeoxyuridine, ribavirin, trisodium phosphophonoformate, amantadine, rimantadine, (*S*)-9-(2,3-Dihydroxypropyl)-adenine, 4',6-dichloroflavan, AZT, 3'(-azido-3'-deoxythymidine), ganciclovir, didanosine (2',3'-dideoxyinosine or ddI),

zalcitabine (2',3'-dideoxycytidine or ddC), dideoxyadenosine (ddA), nevirapine, inhibitors of the HIV protease, and other viral protease inhibitors. Conventional dosages of these agents can be used.

Brief Description of the Drawings

FIGURE 1 Protection of CD3ε+ T-cells against oxidative inhibition by DPI. Lymphocytes and MO were recovered from peripheral blood as described herein. A mixture of MO and lymphocytes was treated with culture medium (control, open circles) or IL-2 (100 U/ml; filled circles) for 16 hrs. After incubation, lymphocytes were labeled with antibodies to CD3ε and CD69. Data show CD69 expression in viable T-cells (CD3ε+) (left panel), and the percentage of T-cells with reduced forward and increased side angle scatter characteristic of apoptosis (right panel). Similar results were obtained when CD69 expression was examined in CD56+. NK-cells incubated with MO: 29.5 % (control) or 79.0 % (DPI 1000 nM) of NK-cells acquired the CD69 antigen in response to IL-2. DPI did not increase the IL-2-induced expression of CD69 in T-cells or NK-cells incubated in the absence of MO (not shown). The results are representative of three similar experiments.

Detailed Description of the Invention

The invention relates to methods of treating cancer or viral diseases with a ROM inhibitory compound is administered alone or in conjunction with additional agents. A ROM inhibitory compound is any compound of composition that inhibits the production and/or release of ROM. The term "ROM inhibitory compound" further encompasses ROM scavengers. The administration of these various agents results in the activation and protection of cytotoxic lymphocytes from the deleterious and inhibitory effects of monocytes/macrophages, as well as a stimulation of the anti-cancer and anti-viral properties of these cells. In addition, the administration of a ROM inhibitory compound in the presence of a vaccine composition results in an increase in lymphocyte proliferation in the presence of monocytes. The addition of other agents that are cytotoxic lymphocyte activation compounds is also contemplated. Cytotoxic lymphocytes are lymphocyte that

possess cytotoxic capabilities such as NK-cells and cytotoxic T-cells (CTLs). The term cytotoxic lymphocytes also encompasses non-cytotoxic cells such as T-helper cells that assist in the activation of a lymphocyte with cytotoxic capabilities. Cytotoxic lymphocyte activation compounds, including those that have an immunological stimulatory character, preferably function in a synergistic fashion with a ROM inhibitory compound. Representatives of such immunological stimulatory compounds include cytokines, peptides, flavonoids, antigens generally, vaccines, and vaccine adjuvants. Additional classes of agents usable with the methods of the invention encompass chemotherapeutic and/or antiviral agents. The methods of the invention are useful for treating neoplastic as well as viral disease.

In contemplating the treatment of individuals suffering from various neoplastic and viral diseases, the invention seeks to stimulate and enhance cell-mediated immunity to accomplish that end. Cell-mediated immunity (CMI) comprises the cytotoxic lymphocyte-mediated immune response to a "foreign agent." The CMI response differs from the antibody-mediated humoral immunity in that the active agent in CMI is a cytotoxic lymphocyte rather than an antibody protein.

Cell-mediated immunity (CMI) operates with cytotoxic lymphocytes such as NK-cells and/or T-cells (CTLs) recognizing and destroying cells displaying "foreign" antigens on their surface. In the invention a foreign agent may be a neoplastic cell or a virus infected cell. As such, CMI functions to eliminate foreign cells from the body. For example, CMI would target cells infected with a virus, rather than to prevent the infection of the cell. Cell-mediated immunity, unlike humoral immunity which can be effective to prevent viral infection, remains the principal mechanism of defense against established viral infections. It is also pivotal in combating neoplastic disease. Therefore, the cytotoxic lymphocyte activity enhancing aspects of the invention are uniquely suited to combat neoplastic and viral diseases.

As discussed above, the immune system contains a number of different cell types, each of which serve to protect the body for foreign invasion. Certain cells of the immune system produce reactive oxygen metabolite (ROM) such as hydrogen peroxide, hypohalous acids, and hydroxyl radicals toward this goal. In previous observations, activation of human natural killer (NK)-cells, a type of cytotoxic lymphocytes, in

response to *in vitro* cytokine stimulation (e.g., IL-2 or IFN-α) is effectively inhibited by autologous monocytes/macrophages (MO). (For review see, Hellstrand, K., *et al.*, Scand. J. Clin. Lab Invest. 57:193-202 (1997)). The inhibitory signal is conveyed by hydrogen peroxide or other reactive oxygen metabolites (ROM) generated by MO. (See Hellstrand, K., *et al.*, J. Immunol., 153: 4940-4947 (1994); Hansson, M., *et al.*, J. Immunol. 156:42-47 (1996)). Addition of hydrogen peroxide scavengers which reduce the concentration of hydrogen peroxide and/or the addition of compounds which inhibit the release of hydrogen peroxide, such as histamine or H_2-receptor agonists, both have been shown to remove the inhibitory effects of MO. *Id.*

T-cells are considered important effector cells responsible for the antitumor properties of various cytokines such as IFN-α and IL-2, observed in experimental tumor models and in human neoplastic disease. (Sabzevari, H., *et al.*, Cancer Res. 53: 4933-4937, (1993); Hakansson, A., *et al.*, Br. J. Cancer, 74: 670-676, (1996); Wersall and Mellstedt, Med. Oncol., 12: 69-77, (1995)). The invention relates, in part, to methods where compounds which reduce the concentration of ROM are used in conjunction with one or more T-cell activation compounds that result in T-cell activation or stimulation. The invention, through the administration of ROM affecting compounds, T-cell activating compounds, and/or anticancer and antiviral compounds, provides methods to treat neoplastic disorders as well as viral infections by increasing the number and specific activity of T-cells.

A number of cytotoxic lymphocyte activation compounds are known in the art to activate and stimulate cytotoxic lymphocyte activity. The dosing, routes of administration and protocols for the use and administration of these materials can be the conventional ones, well known in the art. Generally, interleukins, cytokines and flavonoids have been shown to stimulate cytotoxic lymphocyte activity. Examples of suitable compounds are selected from the group consisting of IL-1, IL-2, IL-12, IL-15, IFN-α, IFN-β, IFN-γ and flavone acetic acid, xanthenone-4-acetic acid, and analogues or derivatives thereto.

Certain vaccines and vaccine adjuvants may also be considered cytotoxic lymphocyte activating compounds. Compounds contemplated here include a number of vaccines and vaccine adjuvants that assist administered antigens to induce rapid, potent,

and long-lasting cytotoxic lymphocyte-mediated immune responses, from immunized or vaccinated individuals. Illustrative vaccines include influenza vaccines, human immunodeficiency virus vaccines, *Salmonella enteritidis* vaccines, hepatitis B vaccines, *Boretella bronchiseptica* vaccines, and tuberculosis vaccines, as well as various anticancer therapeutic vaccines such as allogeneic cancer and autologous cancer vaccines which are known in the art.

The invention is also directed toward the use of a variety of vaccine adjuvants. Such agents including bacillus Calmette-Guerin (BCG), pertussis toxin (PT), cholera toxin (CT), *E. coli* heat-labile toxin (LT), mycobacterial 71-kDa cell wall associated protein, the vaccine adjuvant oil-in-water microemulsion MF59, microparticles prepared from the biodegradable polymers poly(lactide-co-glycolides) (PLG), immune stimulating complexes (iscoms) which are 30-40 nm cage-like structures, (which consist of glycoside molecules of the adjuvant Quil A, cholesterol and phospholipids in which antigen can be integrated), as well as other suitable compounds and compositions known in the art. Such compounds may be administered in amounts sufficient to elicit an effective immune response from an immunized individual.

The invention contemplates and discloses a number of different cytotoxic lymphocyte activating compounds. These compounds may be used to form cytotoxic lymphocyte activating compositions that may be administered as a step of the invention to achieve the activation of a patient's cytotoxic lymphocytes. The invention contemplates the use of the terms cytotoxic lymphocyte activating compound and cytotoxic lymphocyte activation compositions as interchangeable. The dosing, routes of administration and protocols for the use and administration of these materials can be the conventional ones, well known in the art.

The term "reactive oxygen metabolite inhibitors" encompasses a number of disparate compounds. NADPH inhibitors, H_2-receptor agonists, and other compounds with H_2-receptor agonist activity are suitable for use in the invention are known in the art. Examples of suitable compounds include diphenylionodonium (DPI), histamine, compounds with a chemical structure resembling that of histamine or serotonin, yet do not negatively affect H_2-receptor activities. Suitable compounds are selected from the group consisting DPI, histamine, dimaprit, clonidine, tolazoline, impromadine, 4-

methylhistamine, betazole, histamine congeners, H_2-receptor agonists, 8-OH-DPAT, ALK-3, BMY 7378, NAN 190, lisuride, d-LSD, flesoxinan, DHE, MDL 72832, 5-CT, DP-5-CT, ipsapirone, WB 4101, ergotamine, buspirone, metergoline, spiroxatrine, PAPP, SDZ (-) 21009, and butotenine.

A variety of hydrogen peroxide (H_2O_2) scavengers effective to catalyze the decomposition of intercellular H_2O_2 are also known in the art. Suitable compounds are selected from the group consisting of catalase, glutathione peroxidase, ascorbate peroxidase, vitamin E, selen, glutathion, and ascorbate.

Administration of the compounds discussed above can be practiced *in vitro* or *in vivo*. When practiced *in vitro*, any sterile, non-toxic route of administration may be used. When practiced *in vivo*, administration of the compounds discussed above may be achieved advantageously by subcutaneous, intravenous, intramuscular, intraocular, oral, transmucosal, or transdermal routes, for example by injection or by means of a controlled release mechanism. Examples of controlled release mechanisms include polymers, gels, microspheres, liposomes, tablets, capsules, suppositories, pumps, syringes, ocular inserts, transdermal formulations, lotions, creams, transnasal sprays, hydrophilic gums, microcapsules, inhalants, and colloidal drug delivery systems.

The compounds of the invention are administered in a pharmaceutically acceptable form and in substantially non-toxic quantities. A variety of forms of the compounds administered are contemplated by the invention. The compounds may be administered in water with or without a surfactant such as hydroxypropyl cellulose. Dispersions are also contemplated, such as those utilizing glycerol, liquid polyethylene glycols, and oils. Antimicrobial compounds may also be added to the preparations. Injectable preparations may include sterile aqueous solutions or dispersions and powders which may be diluted or suspended in a sterile environment prior to use. Carriers such as solvents or dispersion media contain water, ethanol polyols, vegetable oils and the like may also be added to the compounds of the invention. Coatings such as lecithins and surfactants may be used to maintain the proper fluidity of the composition. Isotonic agents such as sugars or sodium chloride may be added, as well as products intended to delay absorption of the active compounds such as aluminum monostearate and gelatin. Sterile injectable solutions are prepared according to methods well known to those of skill

in the art and can be filtered prior to storage and/or use. Sterile powders may be vacuum or freeze dried from a solution or suspension. Sustained-release preparations and formulations are also contemplated by the invention. Any material used in the composition of the invention should be pharmaceutically acceptable and substantially non-toxic in the amounts employed.

Although in some of the experiments that follow the compounds are used at a single concentration, it should be understood that in the clinical setting, the compounds may be administered in multiple doses over prolonged periods of time. Typically, the compounds may be administered for periods up to about one week, and even for extended periods longer than one month or one year. In some instances, administration of the compounds may be discontinued and then resumed at a later time. A daily dose of the compounds may be administered in several doses, or it may be given as a single dose.

In addition, the compounds of the invention can be administered separately or as a single composition (combined). If administered separately, the compounds should be given in a temporally proximate manner, e.g., within a twenty-four hour period, such that the activation of cytotoxic lymphocytes by the cytokine or other compound is enhanced. More particularly, the compounds may be given within 1 hour of each other. The administration can be by either local or by systemic injection or infusion. Other methods of administration may also be suitable.

The invention also contemplates combinations of cytotoxic lymphocytes activation compounds with ROM production or release inhibiting compounds, ROM scavenging compounds, anticancer compounds, and combinations of antiviral compounds. The doses, routes of administration, and protocols for the use and administration of these materials can be the conventional ones, well known in the art. For example, in one embodiment, IL-2 and IL-12 are combined to activate a population of cytotoxic lymphocytes. In an alternative embodiment, a vaccine or an adjuvant could be used to activate a population of T-cells. In another embodiment, DPI is combined with histamine to inhibit the production or release of hydrogen peroxide from monocytes during a treatment regime. Combinations of various ROM inhibitor compounds, including hydrogen peroxide scavengers such as catalase and ascorbate peroxidase for example, are also contemplated. The invention further contemplates using combinations of all of the

various compounds discussed above to prepare an effective means to stimulate cytotoxic lymphocytes against neoplastic and/or viral disease.

All compound preparations are provided in dosage unit forms for uniform dosage and ease of administration. Each dosage unit form contains a predetermined quantity of active ingredient calculated to produce a desired effect in association with an amount of pharmaceutically acceptable carrier. Such a dosage would therefore define an effective amount of a particular compound.

A preferred compound dosage range can be determined using techniques known to those having ordinary skill in the art. IL-2, IL-12 or IL-15 can be administered in an amount of from about 1,000 to about 600,000 U/kg/day (18 MIU/m^2/day or 1 mg/m^2/day); more preferably, the amount is from about 3,000 to about 200,000 U/kg/day, and even more preferably, the amount is from about 5,000 to about 10,000 U/kg/day.

IFN-α, IFN-β, and IFN-γ can also be administered in an amount of from about 1,000 to about 600,000 U/kg/day; more preferably, the amount is from about 3,000 to about 200,000 U/kg/day, and even more preferably, the amount is from about 10,000 to about 100,000 U/kg/day.

Flavonoid compounds can be administered in an amount of from about 1 to about 100,000 mg/day; more preferable, the amount is from about 5 to about 10,000 mg/day, and even more preferably, the amount is from about 50 to about 1,000 mg/day.

Commonly used doses for the compounds of the invention fall within the ranges listed herein. For example, IL-2 is commonly used alone in doses of about 300,000 U/kg/day. IFN-α is commonly used at 45,000 U/kg/day. IL-12 has been used in clinical trials at doses of 0.5-1.5 μg/kg/day. Motzer, *et al.,* Clin. Cancer Res. 4(5):1183-1191 (1998). IL-1 beta has been used at 0.005 to 0.2 μg/kg/day in cancer patients. Triozzi, *et al.*, J. Clin. Oncol. 13(2):482-489 (1995). IL-15 has been used in rates in doses of 25-400 μg/kg/day. Cao, *et al.,* Cancer Res 58(8):1695-1699 (1998).

Vaccines and vaccine adjuvants can be administered in amounts appropriate to those individual compounds to activate cytotoxic lymphocytes. Appropriate doses for each can readily be determined by techniques well known to those of ordinary skill in the art. Such a determination will be based, in part, on the tolerability and efficacy of a

particular dose using techniques similar to those used to determine proper chemotherapeutic doses.

Compounds effective to inhibit the release or formation of intercellular hydrogen peroxide, or scavengers of hydrogen peroxide, can be administered in an effective amount from about 0.05 to about 10 mg/day; more preferable, the amount is from about 0.1 to about 8 mg/day, and even more preferably, the amount is from about 0.5 to about 5 mg/day. Alternatively, these compounds may be administered from 1 to 100 micrograms per kilogram of patient body weight (1 to 100 μg/kg). However, in each case, the dose depends on the activity of the administered compound. The foregoing doses are appropriate and effective for NADPH inhibitors such as DPI, histamine, H_2-receptor agonists, other intercellular H_2O_2 production or release inhibitors or H_2O_2 scavengers. Appropriate doses for any particular host can be readily determined by empirical techniques well known to those of ordinary skill in the art.

The invention contemplates identifying a patient in need of enhanced cytotoxic lymphocyte activity and increasing that patient's circulating blood ROM inhibitory compound concentration to an optimum, beneficial, therapeutic level so as to provide for more efficient cytotoxic lymphocyte stimulation. Such a level may be achieved through repeated injections of the compounds of the invention in the course of a day, during a period of treatment.

Subjects suffering from cancer often exhibit decreased levels of circulating blood histamine. (Burtin *et al.* Decreased blood histamine levels in subjects with solid malignant tumors, Br. J. Cancer 47: 367-372 (1983)). Thus, the elevation of blood histamine concentrations to beneficial levels finds ready application to cancer and antiviral treatments based on synergistic effects between histamine and agents which enhance cytotoxic effector cell mediated cytotoxicity. In such protocols, the activity of T-cells is enhanced. For example, the cytotoxic activity of cytotoxic T lymphocytes (CTLs) is enhanced by combining the administration of a H_2-receptor agonist such as histamine to increase circulating histamine to a beneficial level sufficient to augment the activity of an agent which acts in synergy with a H_2-receptor agonist to increase cytotoxicity with the administration of the agent.

In one embodiment of the invention, beneficial levels of circulating blood ROM inhibitory compound levels, such as DPI or H_2-receptor agonist are obtained by administering the ROM inhibitory compound at a dosage of 0.05 to 10 mg/day. In a another embodiment, beneficial blood levels of ROM inhibitory compounds are administered at 1 to 100 microgram per kilogram of patient body weight (1 to 100 μg/kg). In an another embodiment, the ROM inhibitory compound is administered over a treatment period of 1 to 4 weeks with injections occurring as frequently as several times daily, over a period of up to 52 weeks. In still another embodiment, the ROM inhibitory compound is administered for a period of 1-2 weeks, with multiple injections occurring as frequently as several times daily. This administration can be repeated every few weeks over a time period of up to 52 weeks, or longer. Additionally, the frequency of administration may be varied depending on the patient's tolerance of the treatment and the success of the treatment. For example, the administrations may occur three times per week, or even daily, for a period of up to 24 months.

One embodiment the invention contemplates utility with respect to the treatment of various cancers or neoplastic diseases. Malignancies against which the invention may be directed include, but are not limited to, primary and metastatic malignant tumor disease, hematological malignancies such as acute and chronic myelogenous leukemia, acute and chronic lymphatic leukemia, multiple myeloma, Waldenstroms Macroglobulinemia, hairy cell leukemia, myelodysplastic syndrome, polycytaemia vera, and essential thrombocytosis.

The method of the invention may also be utilized alone or in combination with other anticancer therapies. When used in combination with a chemotherapeutic regime, a ROM inhibitory compound and a cytotoxic lymphocyte activating compound are administered with a chemotherapeutic agent or agents. The doses, routes of administration and protocols for the use and administration of these materials can be the conventional ones, well known in the art. Representative compounds used in cancer therapy include cyclophosphamide, chlorambucil, melphalan, estramustine, iphosphamide, prednimustin, busulphan, tiottepa, carmustin, lomustine, methotrexate, azathioprine, mercaptopurine, thioguanine, cytarabine, fluorouracil, vinblastine, vincristine, vindesine, etoposide, teniposide, dactinomucin, doxorubin, dunorubicine, epirubicine, bleomycin,

nitomycin, cisplatin, carboplatin, procarbazine, amacrine, mitoxantron, tamoxifen, nilutamid, and aminoglutemide. Procedures for employing these compounds against malignancies are well established. In addition, other cancer therapy compounds may also be utilized with the invention.

The invention contemplates treatment of a variety of viral diseases. The following are merely examples of some of the viral diseases against which the invention is effective. There are a number of herpetic diseases caused by herpes simplex or herpes zoster viruses including herpes facialis, herpes genitalis, herpes labialis, herpes praeputialis, herpes progenitalis, herpes menstrualis, herpetic keratitis, herpes encephalitis, herpes zoster ophthalmicus, and shingles. The invention is effective as a treatment against each of these diseases.

Another aspect of the shows the invention to be effective against viruses that cause diseases of the enteric tract such as rotavirus mediated disease.

In another aspect, the invention is effective against various blood based infections. For example, yellow fever, dengue, ebola, Crimean-Congo hemorrhagic fever, hanta virus disease, mononucleosis, and HIV/AIDS.

Another aspect of the invention is directed toward various hepatitis causing viruses. A representative group of these viruses includes hepatitis A virus, hepatitis B virus, hepatitis C virus, hepatitis D virus, and hepatitis E virus.

In still another aspect, the invention is effective against respiratory tract diseases caused by viral infections. Examples include: rhinovirus infection (common cold), mumps, rubella, varicella, influenza B, respiratory syncytial virus infection, measles, acute febrile pharyngitis, pharyngoconjunctival fever, and acute respiratory disease.

Another aspect of the invention contemplates treatment for various cancer linked viruses, including: adult T-cell leukemia/lymphoma (HTLVs), nasopharyngeal carcinomas, Burkitt's lymphoma (EBV), cervical carcinomas, hepatocellular carcinomas.

In still a further aspect, the invention is useful in the treatment of viral-meditated encephalitis, including: St. Louis encephalitis, Western encephalitis, and tick-borne encephalitis.

The methods of the invention may also be utilized alone or in combination with other antiviral therapies. When used in combination with an antiviral chemotherapeutic regime, a ROM inhibitory compound, and the cytotoxic lymphocyte activating compound are administered with an antiviral chemotherapeutic agent or agents. The doses, routes of administration and protocols for the use and administration of these materials can be the conventional ones, well known in the art. Representative compounds used in antiviral chemotherapy include idoxuridine, trifluorothymidine, adenine arabinoside, acycloguanosine, bromovinyldeoxyuridine, ribavirin, trisodium phosphophonoformate, amantadine, rimantadine, (*S*)-9-(2,3-Dihydroxypropyl)-adenine, 4',6-dichloroflavan, AZT, 3'(-azido-3'-deoxythymidine), ganciclovir, didanosine (2',3'-dideoxyinosine or ddI), zalcitabine (2',3'-dideoxycytidine or ddC), dideoxyadenosine (ddA), nevirapine, inhibitors of the HIV protease, and other viral protease inhibitors.

The invention also contemplates using a combination of anticancer and antiviral agents in conjunction with the administration of a ROM inhibitory compound.

Although not intended to limit the invention, it is contemplated that the methods of the invention augment cytotoxic lymphocyte activity by altering the mechanics of antigen presentation. One theory provides that monocytes/macrophages that are also antigen presenting cells (APC) are inhibited from presenting antigens to T-cells. This inhibition might result from MO metabolic pathways dedicated to the generation of ROM that inhibit MO antigen presenting metabolic pathways, producing mutually exclusive antigen presenting or ROM producing states in MO populations. A result of the inhibition of MO antigen presentation is that T-cell populations would remain dormant in the absence of presented antigen and in the presence of ROM.

Under this theory, administration of ROM production and release inhibiting compounds, such as histamine, acts to increase T-cell activity by increasing antigen presentation. Monocytes producing ROM may have a molecular switch thrown in the present of beneficial concentrations of histamine that results in a down regulation of ROM production. In the mutually exclusive metabolic state hypothesized above, the down regulation of ROM production results in a subsequent increase in antigen presentation pathways and thus antigen presentation. Accordingly, administration of histamine in the

presence of an antigen based T-cell activator, like a vaccine, would serve to increase T-cell activity by decreasing ROM production and increasing antigen presentation.

In an alternative theory, the administration of a ROM inhibitory compound, results in an increase cytotoxic lymphocyte activity by removing ROM induced cytotoxic lymphocyte inhibition.

The examples discussed below apply the teachings of the invention and show that monocytes/macrophages (MO), and particularly MO-derived reactive oxygen metabolites (ROMs), effectively suppress the activation of human cytotoxic lymphocytes even after the *in vitro* administration of cytotoxic lymphocyte activation compounds such IFN-α or IL-2. Furthermore, it is shown that the addition of a ROM inhibitory compound confers protection to cytotoxic lymphocytes when added to a mixture of lymphocytes and MO.

To determine the effect of the various compounds of the invention on a population of T-cells, the expression of various cytotoxic lymphocyte markers that are inducibly expressed on the surface of mature human cytotoxic lymphocytes was studied. The observed results show that cytokine-induced activation of cytotoxic lymphocytes, as reflected by the appearance of CD69 or other markers after incubation with representative cytokines such as IL-2 or IFN-α, was profoundly inhibited by MO in the absence of a ROM inhibitory compound. However, addition of such ROM inhibitory compounds effectively reversed the observed inhibitory effects of MO. Additional work was performed to study the effect of histamine on the proliferative response of human cytotoxic lymphocytes to a polyvalent vaccine against influenza virus *in vitro*. The administration of histamine in these experiments was shown to elevate lymphocyte proliferation in presence of antigen and monocytes.

EXAMPLES

Particular aspects of the invention can be more readily understood by reference to the following examples, which are intended to exemplify the invention, without limiting its scope to the particular exemplified embodiments.

The methods of the invention may be used to enhance the activation and protection of cytotoxic lymphocyte populations using various cytotoxic lymphocyte

activation compounds that result in cytotoxic lymphocyte stimulation and/or activation. ROM inhibitory compounds such as NADPH inhibitors, H_2-receptor agonists, and H_2O_2 scavengers and inhibitors are discussed below. To demonstrate the activation and protection characteristics of these compounds, lymphocytes (including NK-cells and T-cells) and monocytes were isolated from donated blood and examined for the activation characteristics when exposed various cytotoxic lymphocyte activating compounds, such as IL-2 and/or IFN-α, vaccines, vaccine adjuvants or other immunological stimulator compounds, various ROM inhibitory compounds, such as DPI, histamine, and various H_2O_2 scavengers, such as catalase.

Peripheral venous blood was obtained as freshly prepared leukopacks from healthy blood donors at the Blood Centre, Sahlgren's Hospital, Göteborg, Sweden, to study the activation characteristics of cytotoxic lymphocytes in the presence and absence of MO, and ROM inhibitors. The blood (65 ml) was mixed with 92.5 ml Iscove's medium, 35 ml 6% Dextran (Kabi Pharmacia, Stockholm, Sweden) and 7.5 ml acid citrate dextrose (ACD) (Baxter, Deerfield, Illinois). After incubation for 15 minutes at room temperature, the supernatant was carefully layered onto Ficoll-Hypaque (Lymphoprep, Myegaard, Norway). Mononuclear cells (MNC) were collected at the interface after centrifugation at 380 *g* for 15 minutes at room temperature, washed twice in PBS and resuspended in Iscove's medium supplemented with 10 % human AB^+ serum. During all further separation of cells, the cell suspensions were kept in siliconized test tubes (Vacuette, Greiner, Stockholm).

The MNC were further separated into lymphocyte and monocyte (MO) populations using the counter-current centrifugal elutriation (CCE) technique originally described by Yasaka and co-workers (Yasaka, T. *et al.*, J. Immunol., 127:1515) with modifications as described in Hansson, M., *et al.* (J. Immunol., 156: 42 (1996); hereby incorporated by reference). Briefly, the MNC were resuspended in elutration buffer containing 0.05% BSA and 0.015% EDTA in buffered NaCl and fed into a Beckman J2-21 ultracentrifuge with a JE-6B rotor at 2100 rpm. A fraction with >90 % MO was obtained at a flow rate of 18 ml/min. A lymphocyte fraction enriched for NK-cells ($CD3^-$ / 56^+ phenotype) and T-cells ($CD3^+$ / 56^-) was recovered at flow rates of 14-15 ml/min. This fraction contained <3% MO and consisted of $CD3\epsilon^-$ / 56^+ NK-cells (45-

50%), $CD3\epsilon^+$ / 56^- T-cells (35-40%), $CD3\epsilon^-$ / 56^- cells (5-10%), and $CD3\epsilon^+$ / 56^+ cells (1-5%), as judged by flow cytometry. In some experiments, dynabeads (Dynal A/S, Oslo, Norway) coated with anti-CD56 were used to obtain purified lymphocyte preparations of T-cells, as described in detail by Hansson, M., *et al.*, incorporated above.

Following fractionation, the lymphocyte mixture of T-cells and NK cells was exposed to the various experimental conditions described below and assayed for activation using the appearance of certain cell surface proteins as indicia of activation.

Lymphocytes are identifiable by certain proteins which reside on the cell surface. Different cell surface proteins reside on different classes of lymphocytes and lymphocytes in different stages of activation. These proteins have been grouped into CD classes or "clusters of differentiation" and may serve as markers for different types of cells. Labeled antibodies, specific for different cell surface proteins, that bind to the different CD markers may be used to identify the different types of T-cells and their respective states of activation.

In the experiments described below, CD3, CD4, CD8, CD69 and CD56 (a NK-cell marker), were used to identify the cytotoxic lymphocytes of interest. The CD3 group of antibodies is specific for a marker expressed on all peripheral T-cells. The CD4 group of antibodies is specific for a marker on class II MHC-restricted T-cells, also known as T helper cells. The CD8 group of antibodies recognize a marker on class I MHC-restricted T-cells, also known as CTLs or cytolytic T-cells. The CD69 group of antibodies recognizes activated T-cells and other activated immune cells. Finally, the CD56 groups recognizes a heterodimer on the surface of NK-cells.

Flow cytometry was used in the experiments described below to identify the various sub-populations of T-cells. Flow cytometry permits an investigator to examine a population of cells using a number of labeled probes to differentiate sub-populations within the larger whole. In these experiments, the CD3 marker was used to identify the sub-population of T-cells and the CD4 and CD8 markers were used to further identify the sub-population of T-cells into T helper cells and CTLs. The effects of MO exposure in the presence and absence of histamine and T-cell activation compounds were determined using the CD69 T-cell activation marker. The expression of the different

markers was estimated in a lymphocyte gate using flow cytometry (as described in Hellstrand, K., *et al.* Cell. Immunol. 138: 44-54 (1991), and hereby incorporated by reference).

The following protocol was used in experiments reporting the detection of surface antigens of cell populations. One million cells were incubated with appropriate fluorescein isothiocynate (FITC) and phycoerythrin (PE) conjugated monoclonal antibodies (Becton & Dickinson, Stockholm, Sweden; 1 μl/10^6 cells), on ice for 30 minutes. The cells were washed twice in PBS and resuspended in 500 μl sterile filtered PBS and analyzed by use of flow cytometry on a FACSort with a Lysys II software program (Becton & Dickenson). Lymphocytes were gated on the basis of forward and right angle scatter. The flow rate was adjusted to <200 cells x s^{-1} and at least 5 x 10^3 cells were analyzed for each sample, if not otherwise stated.

EXAMPLES

Particular aspects of the invention can be more readily understood by reference to the following examples, which are intended to exemplify the invention, without limiting its scope to the particular exemplified embodiments.

The methods of the invention may be used to enhance the activation and protection of cytotoxic lymphocyte populations using various cytotoxic lymphocyte activation compounds that result in cytotoxic lymphocyte stimulation and/or activation. ROM inhibitory compounds such as DPI, are discussed below.

To demonstrate the activation and protection characteristics of these compounds, lymphocytes (including NK-cells and T-cells) and monocytes were isolated from donated blood and examined for the activation characteristics when exposed various cytotoxic lymphocyte activating compounds, such as IL-2 and/or IFN-α, vaccines, vaccine adjuvants or other immunological stimulator compounds, various ROM inhibitory compounds, such as DPI, histamine, and various H_2O_2 scavengers, such as catalase.

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EXAMPLE 1

To assess whether DPI and histamine inhibited the spontaneous release of ROM from MO, a chemiluminescence assay was performed that specifically quantified extracellular ROM (superoxide anion). The spontaneous isoluminol-enhanced extracellular generation of superoxide anion in elutriated MO was measured using the assay described in Hellstrand, K., et al., J Immunol, 153:4940-4947 (1994) and in Lundqvist & Dahlgren, *Free Radic. Biol. Mod.* 20:785-792 (1996). A more than four fold reduction of released extracellular superoxide anion was observed at DPI concentrations of 10 nM, and similar results were obtained in three experiments using MO recovered from three blood donors. Similarly, histamine (at 50 μM) inhibited the concentration of extracellular superoxide anion in this model more than five-fold. This effect of histamine was completely antagonized by ranitidine, used at concentrations equimolar to histamine.

EXAMPLE 2

MO can reduce molecular oxygen and generate ROM (respiratory burst), both spontaneously and in response to certain soluble or particulate stimuli (see Klebanoff, S. J., *Adv. Host Def. Mech. 1:*111-151 (1982)). Experiments were performed in which DPI, an inhibitor of NADPH oxidase activity in MO (Miesel, R., et al., *Free. Radic. Biol 20:*75-81 (1996)), was added to mixtures of lymphocytes and MO in studies of the acquisition of CD69 on T-cells and NK-cells in response to IL-2. A mixture of MO and lymphocytes was treated with culture medium or IL-2 (100 U/ml) for 16 hours. After incubation, lymphocytes were labeled with antibodies to CD3 ε and CD69. Data show CD69 expression in viable T-cells (CD3 ε +), and the percentage of T-cells with reduced forward and increased side angle scatter characteristic of apoptosis. Similar results were obtained when CD69 expression was examined in CD56+. NK-cells incubated with MO: 29.5 % (control) or 79.0 % (DPI 1000 nM) of NK-cells acquired the CD69 antigen in response to IL-2. DPI did not increase the IL-2-induced expression of CD69 in T-cells or NK-cells incubated in the absence of MO. DPI significantly reversed the MO-induced inhibition of T-cells (FIGURE 1) and NK-cells (not shown). MO also produce reactive nitrogen intermediates of which nitric oxide (NO) is the ultimate effector molecule, and DPI is an inhibitor also of NO synthetase (Miesel, R., et al., *Free. Radic.*

Biol 20:75-81 (1996)). To study whether NO induction in MO contributed to the observed T- and NK-cell anergy to IL-2, we used a NO synthetase inhibitor, N-monomethyl-L-arginine (L-NMMA). This compound, used at concentrations sufficient to inhibit NO synthesis in MO (Hansson, M, et al., J Immunol. 156:42-47 (1996)), did not affect the MO-induced suppression of T-cells and NK-cells. Catalase, a scavenger of hydrogen peroxide, significantly reversed the MO-induced inhibition of IL-2-induced CD69 expression in T-cells and NK-cells at concentrations exceeding 50 U/ml, whereas superoxide dismutase, a scavenger of superoxide anion, was ineffective at concentrations sufficient to scavenge >90 % of superoxide anion (200 U/ml).

EXAMPLE 3

The Fas ligand (CD95L) triggers apoptosis in many cell types after interaction with the Fas receptor (CD95), which is expressed on *inter alia* T cells (Alderson, M. R., et al., *J Exp. Mod. 181:*71-77(1995)), and NK-cells (Medvedev, A. E., et al., *Cytokine* 9:394-404 (1997)). To evaluate the role of FasL/Fas interactions for the observed oxidatively induced apoptosis, a Fas ligand inhibitor was used that comprised the extracellular domain of human Fas (aa 1-154), fused to the Fc portion of human IgG1. This Fas:Fc-IgG fusion protein, used at a concentration (20 μg/ml) sufficient to reduce FasL-mediated, activation-induced apoptosis in T-cells by >60 %, did not affect the MO-induced anergy to IL-2 or the MO-induced apoptosis in T-cells or NK-cells (Table 1).

TABLE 1

Fas/FasL-independent anergy and apoptosis in T-cells and NK-cells.

	viable CD3+/CD69+	viable CD56+/69+	apoptotic	apoptotic
treatment	(gated events)[A]	(gated events)	CD3+/CD69+(%)[B]	CD56+/CD69+(%)
control	89	36	52	71
DPI	701	1248	7	13
Fas; Fc-IgG	113	26	57	68

[A]Lymphocytes and MO were recovered from peripheral blood and labelled with anti-CD3ε, anti-CD56, and anti-CD69 as described in the legend to table I and analyzed for respective phenotype by flow cytometry. DPI was used at 100 nM and Fas:Fc-IgG at 20 μg/ml. [B]Apoptosis was measured by flow cytometry using a gate for lymphocytes with reduced

forward scatter and increased right angle scatter. Similar results were obtained in three separate experiments.

EXAMPLE 4

DPI, in a dose approximately 0.2 to 2.0 mg or 3-10 μg/kg, in a pharmaceutically acceptable form is injected subcutaneously in a sterile carrier solution into subjects in need of enhanced T-cell activity, in this case a patient having a malignancy. Concomitantly, IL-2, for example, human recombinant IL-2 (Proleukin®, Eurocetus), is administered subcutaneously or by continuous infusion of 27μg/kg/day on days 1-5 and 8-12. This dose represents a total dose of IL-2 considerably lower than that administered by those of skill in the art.

The above procedure is repeated every 4-6 weeks until an objective regression of tumor disease is observed. The therapy may be continued even after a partial or complete response has been observed. In patients with complete responses, the therapy may be given with longer intervals between cycles.

The treatment may also include periodically boosting patient blood DPI levels by administering 0.2 to 2.0 mg or 3-10 μg/kg of DPI injected 1, 2, or more times per day over a period of one to two weeks at regular intervals, such as daily, bi-weekly, or weekly in order to establish blood DPI at a beneficial concentration

Combination of DPI and Chemotherapeutic Agents

DPI may also be used in conjunction with chemotherapeutic agents to treat a neoplastic or viral disease. Monocyte mediated suppression can be eliminated by administration of DPI prior, during, following or throughout chemotherapy in order to facilitate activation and protection of cytotoxic lymphocytes.

Representative compounds used in cancer and antiviral therapies are described above. Other cancer and antiviral therapeutic compounds may also be utilized in the invention. Similarly, malignancies and viral diseases against which the treatment of the invention may be effective and thus may be directed are also described. It should be noted that the amounts, routes of administration and dosage protocols for these cancer and

antiviral compounds used with the methods of the invention are well known to those of skill in the art. The invention is directed toward augmenting the efficacy of these compounds, and the therapeutic results of their use. Therefore, the conventional methodologies for their use, in conjunction with the compounds and methods of the invention, are contemplated as sufficient to achieve a desired therapeutic effect.

A combination of histamine and IL-2 for activating NK cells has proven an effective combination with traditional chemotherapeutic methods in treating acute myelogenous leukemia. Brune and Hellstrand, Br. J. Haematology, 92:620-626 (1996).

EXAMPLE 5

Subjects in need of enhanced cytotoxic lymphocyte activity, because of a neoplastic disease, and/or a viral infection such as hepatitis B (HBV), hepatitis C (HCV), human immunodeficiency virus (HIV), human papilloma virus (HPV) or herpes simplex virus (HSV) type 1 or 2, or other viral infections, are administered human recombinant IL-2 (Proleukin®, Eurocetus) by subcutaneous injection or by continuous infusion of 27μg/kg/day on days 1-5 and 8-12. Additionally, subjects may also receive a daily dose of $6x10^6$ U interferon-α administered by a suitable route, such as subcutaneous injection. This treatment also includes administering 0.2 to 2.0 mg or 3-10 μg/kg of DPI injected 1, 2, or more times per day in conjunction with the administration of IL-2 and/or interferon-α.

The above procedure is repeated every 4-6 weeks until an objective regression of the tumor is observed, or until improvement in the viral infection occurs. The therapy may be continued even after a first, second, or subsequent complete remission has been observed. In patients with complete responses, the therapy may be given with longer intervals between cycles.

The treatment may also include periodically boosting patient blood DPI levels by administering 0.2 to 2.0 mg or 3-10 μg/kg of DPI injected 1, 2, or more times per day over a period of one to two weeks at regular intervals, such as daily, bi-weekly, or weekly in order to establish or maintain blood DPI at a beneficial concentration, e.g., at a concentration above 0.2μmole/L.

Additionally, the frequency of interferon-α administration may be varied depending on the patient's tolerance of the treatment and the success of the treatment. For example, interferon may be administered three times per week, or even daily, for a period of up to 24 months. Those skilled in the art are familiar varying interferon treatments to achieve both beneficial results and patient comfort.

EXAMPLE 6

Subjects with AML in a first, second, subsequent or complete remission are treated in 21-day courses with IL-2 [35-50 μg (equivalent to 6.3-9 x 10^5 IU) subcutaneously (s.c.). twice daily], repeated with three to six-week intermissions and continued until relapse. In cycle #1, patients receive three weeks of low dose chemotherapy consisting of 16mg/m^2/day cytarabine, and 40 mg/day thioguanine. Concomitantly, patients are injected subcutaneously with 0.2 to 2.0 mg or 3-10μg/kg of a pharmaceutically acceptable form of DPI to boost circulating DPI to a beneficial level twice daily (above 0.2 μmole/L). DPI levels may be continually boosted to beneficial levels by administering DPI by injection at 0.2 to 2.0 mg or 3-10 μg/kg twice daily in a pharmaceutically acceptable form of a ROM inhibitory compound during the IL-2 treatment. Thereafter, the subjects are allowed to rest for three to six weeks.

After the rest period at the end of the first cycle (cycle #1), the second cycle (cycle #2) is initiated. Twice daily, injections of a pharmaceutically acceptable form of a ROM inhibitory compound in a sterile carrier solution are administered at 0.5 to 2.0 mg or 3-10 μg/kg subcutaneously. Cytarabine (16 mg/m^2/day s.c.) and thioguanine (40 mg/day orally) are given for 21 days (or until the platelet count is ≤ 50 x 10^9/1). In the middle week, patients receive 0.2 to 2.0 mg or 3-10 μg/kg per injection twice per day of a pharmaceutically acceptable form of DPI to boost circulating DPI to beneficial levels. At the end of the three week chemotherapy treatment, patients receive 0.2 to 2.0 mg or 3-10 μg/kg per injection twice daily of a pharmaceutically acceptable form of DPI for a week. Thereafter, patients receive interleukin-2 for three weeks. Patients are permitted to rest for three to six weeks.

Thereafter, cycle #3 is initiated. Cycle #3 is identical to cycle #2.

Alternatively, the treatment may also include periodically boosting patient blood DPI levels by administering 0.2 to 2.0 mg or 3-10 μg/kg of DPI injected 1, 2, or more times per day over a period of one to two weeks at regular intervals, such as daily, bi-weekly, or weekly in order to achieve a beneficial blood DPI concentration. Another alternative is to provide DPI in a depot or controlled release form.

DISCUSSION

The data presented herein demonstrate that MO inhibit cytotoxic lymphocyte activation. MO inhibition of cytotoxic lymphocyte activation appears to be mediated by ROM formation. The experiments discussed above show that MO inhibition of cytotoxic lymphocyte is reversed through the addition of a ROM inhibitory compound such as DPI. These results suggest that cytotoxic lymphocyte activation may benefit from a down-regulation of MO inhibition.

CONCLUSION

While particular embodiments of the invention have been described in detail, it will be apparent to those of skill in the art that these embodiments are exemplary, rather than limiting. All references are hereby expressly incorporated by reference.

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WHAT IS CLAIMED IS:

1. A method for activating and protecting cytotoxic lymphocytes in the presence of monocytes (MO), comprising:

identifying a subject in need of enhanced cytotoxic lymphocyte activity; and

administering to the patient an amount of diphenylionodonium (DPI), effective to activate and protect cytotoxic lymphocyte function in the presence of MO.

2. The method of Claim 1, further comprising administering an effective amount of a cytotoxic lymphocyte stimulatory composition to the subject, wherein said cytotoxic lymphocyte stimulatory composition is selected from the group consisting of a vaccine adjuvant, a vaccine, a peptide, a cytokine, and a flavonoid.

3. The method of Claim 2, wherein the composition is a vaccine adjuvant selected from the group consisting of bacillus Calmette-Guerin (BCG), pertussis toxin (PT), cholera toxin (CT), *E. coli* heat-labile toxin (LT), mycobacterial 71-kDa cell wall associated protein, microemulsion MF59, microparticles of poly(lactide-co-glycolides)(PLG), and immune stimulating complexes (ISCOMS).

4. The method of Claim 2, wherein the composition is a vaccine selected from the group consisting of influenza vaccines, human immunodeficiency virus vaccines, *Salmonella enteritidis* vaccines, hepatitis B vaccines, *Boretella bronchiseptica* vaccines, tuberculosis vaccines, allogeneic cancer vaccines, and autologous cancer vaccines.

5. The method of Claim 2, wherein the composition is a cytokine selected from the group consisting of IL-1, IL-2, IL-12, IL-15, IFN-α, IFN-β, or IFN-γ.

6. The method of Claim 2, wherein the composition is a flavonoid selected from the group consisting of flavone acetic acids and xanthenone-4-acetic acids.

7. The method of Claim 2, wherein said cytotoxic lymphocyte stimulatory composition is administered in a daily dose of between 1000 and 600,000 U/kg.

8. The method of Claim 1, further comprising administering of an effective amount of a compound that inhibits the production or release of intercellular reactive oxygen metabolites (ROM) selected from the group consisting of histamine, histamine

dihydrochloride, histamine phosphate, serotonin, dimaprit, clonidine, tolazoline, impromadine, 4-methylhistamine, betazole, and a histamine congener.

9. The method of Claim 8, wherein said effective amount is between 0.05 and 50 mg per dose.

10. The method of Claim 8, wherein said effective amount is between 1 and 500 μg/kg of patient weight per dose.

11. The method of Claim 1, wherein the administration of said cytotoxic lymphocyte stimulatory composition and said effective amount of a compound that inhibits the production or release of intercellular reactive oxygen metabolites (ROM) is performed within 1 hour.

12. The method of Claim 1, wherein the administration of said cytotoxic lymphocyte stimulatory composition and said effective amount of a compound that inhibits the production or release of intercellular reactive oxygen metabolites (ROM) is performed within 24 hours.

13. The method of Claim 8, wherein said intercellular reactive oxygen metabolite is hydrogen peroxide.

14. The method of Claim 13, further comprising administering an effective amount of a scavenger of intercellular hydrogen peroxide.

15. The method of Claim 14, wherein the scavenger is selected from the group consisting of catalase, glutathione peroxidase, and ascorbate peroxidase.

16. The method of Claim 14, wherein said hydrogen peroxide scavenger is administered in a dose of from about 0.05 to about 50 mg/day.

17. The method of Claim 14, wherein said effective amount of DPI, said cytotoxic lymphocyte stimulatory composition and said scavenger of hydrogen peroxide are administered separately.

18. The method of claim 1, further comprising the administering a chemotherapeutic agent.

19. The method of claim 18, wherein the chemotherapeutic agent comprises an anticancer agent selected from the group consisting of cyclophosphamide, chlorambucil, melphalan, estramustine, iphosphamide, prednimustin, busulphan, tiottepa, carmustin, lomustine, methotrexate, azathioprine, mercaptopurine, thioguanine,

cytarabine, fluorouracil, vinblastine, vincristine, vindesine, etoposide, teniposide, dactinomucin, doxorubin, dunorubicine, epirubicine, bleomycin, nitomycin, cisplatin, carboplatin, procarbazine, amacrine, mitoxantron, tamoxifen, nilutamid, and aminoglutemide.

20. The method of claim 18, wherein the chemotherapeutic agent comprises an antiviral agent selected from the group consisting of idoxuridine, trifluorothymidine, adenine arabinoside, acycloguanosine, bromovinyldeoxyuridine, ribavirin, trisodium phosphophonoformate, amantadine, rimantadine, (*S*)-9-(2,3-Dihydroxypropyl)-adenine, 4',6-dichloroflavan, AZT, 3'(-azido-3'-deoxythymidine), ganciclovir, didanosine (2',3'-dideoxyinosine or ddI), zalcitabine (2',3'-dideoxycytidine or ddC), dideoxyadenosine (ddA), nevirapine, inhibitors of the HIV protease, and other viral protease inhibitors.

21. The method of claim 18, wherein administering said effective amount of DPI, said cytotoxic lymphocyte stimulatory composition, said compound that inhibits the production or release of intercellular hydrogen peroxide and said chemotherapeutic agent are performed concomitantly.

22. A composition comprising a cytotoxic lymphocyte protecting amount of diphenylionodonium (DPI) in a pharmaceutically acceptable carrier.

23. The composition of Claim 22, further comprising a cytotoxic lymphocyte stimulatory compound selected from the group consisting of a vaccine adjuvant, a vaccine, a peptide, a cytokine, and a flavonoid.

24. The composition of Claim 23, wherein the compound is a vaccine adjuvant selected from the group consisting of bacillus Calmette-Guerin (BCG), pertussis toxin (PT), cholera toxin (CT), *E. coli* heat-labile toxin (LT), mycobacterial 71-kDa cell wall associated protein, microemulsion MF59, microparticles of poly(lactide-co-glycolides)(PLG), and immune stimulating complexes (ISCOMS).

25. The composition of Claim 23, wherein the compound is a vaccine selected from the group consisting of influenza vaccines, human immunodeficiency virus vaccines, *Salmonella enteritidis* vaccines, hepatitis B vaccines, *Boretella bronchiseptica* vaccines, tuberculosis vaccines, allogeneic cancer vaccines, and autologous cancer vaccines.

26. The composition of Claim 23, wherein the compound is a cytokine selected from the group consisting of IL-1, IL-2, IL-12, IL-15, IFN-α, IFN-β, or IFN-γ.

27. The composition of Claim 23, wherein the compound is a flavonoid selected from the group consisting of flavone acetic acids and xanthenone-4-acetic acids.

28. The composition of Claim 23, wherein said cytotoxic lymphocyte stimulatory composition is administered in a daily dose of between 1000 and 600,000 U/kg.

29. The composition of Claim 22, further comprising an effective amount of a compound that inhibits the production or release of intercellular reactive oxygen metabolites (ROM) selected from the group consisting of histamine, histamine dihydrochloride, histamine phosphate, serotonin, dimaprit, clonidine, tolazoline, impromadine, 4-methylhistamine, betazole, and a histamine congener.

30. The composition of Claim 29, wherein said effective amount of a compound that inhibits the production or release of intercellular reactive oxygen metabolites (ROM) is between 0.05 and 50 mg per dose.

31. The composition of Claim 29, wherein said effective amount of a compound that inhibits the production or release of intercellular reactive oxygen metabolites (ROM) is between 1 and 500 μg/kg of patient weight per dose.

32. The composition of claim 22, further comprising a chemotherapeutic agent.

33. The composition of claim 32, wherein the chemotherapeutic agent comprises an anticancer agent selected from the group consisting of cyclophosphamide, chlorambucil, melphalan, estramustine, iphosphamide, prednimustin, busulphan, tiottepa, carmustin, lomustine, methotrexate, azathioprine, mercaptopurine, thioguanine, cytarabine, fluorouracil, vinblastine, vincristine, vindesine, etoposide, teniposide, dactinomucin, doxorubin, dunorubicine, epirubicine, bleomycin, nitomycin, cisplatin, carboplatin, procarbazine, amacrine, mitoxantron, tamoxifen, nilutamid, and aminoglutemide.

34. The composition of claim 32, wherein the chemotherapeutic agent comprises an antiviral agent selected from the group consisting of idoxuridine,

trifluorothymidine, adenine arabinoside, acycloguanosine, bromovinyldeoxyuridine, ribavirin, trisodium phosphophonoformate, amantadine, rimantadine, (*S*)-9-(2,3-Dihydroxypropyl)-adenine, 4',6-dichloroflavan, AZT, 3'(-azido-3'-deoxythymidine), ganciclovir, didanosine (2',3'-dideoxyinosine or ddI), zalcitabine (2',3'-dideoxycytidine or ddC), dideoxyadenosine (ddA), nevirapine, inhibitors of the HIV protease, and other viral protease inhibitors.

ACTIVATION AND PROTECTION OF CYTOTOXIC LYMPHOCYTES USING A REACTIVE OXYGEN METABOLITE INHIBITOR

Abstract of the Disclosure

Methods and compositions for activating and protecting cytotoxic lymphocytes in the presence of monocytes (MO), comprising identifying a subject in need of enhanced cytotoxic lymphocyte activity; and administering to the patient an amount of diphenylionodonium (DPI), effective to activate and protect cytotoxic lymphocyte function in the presence of MO.

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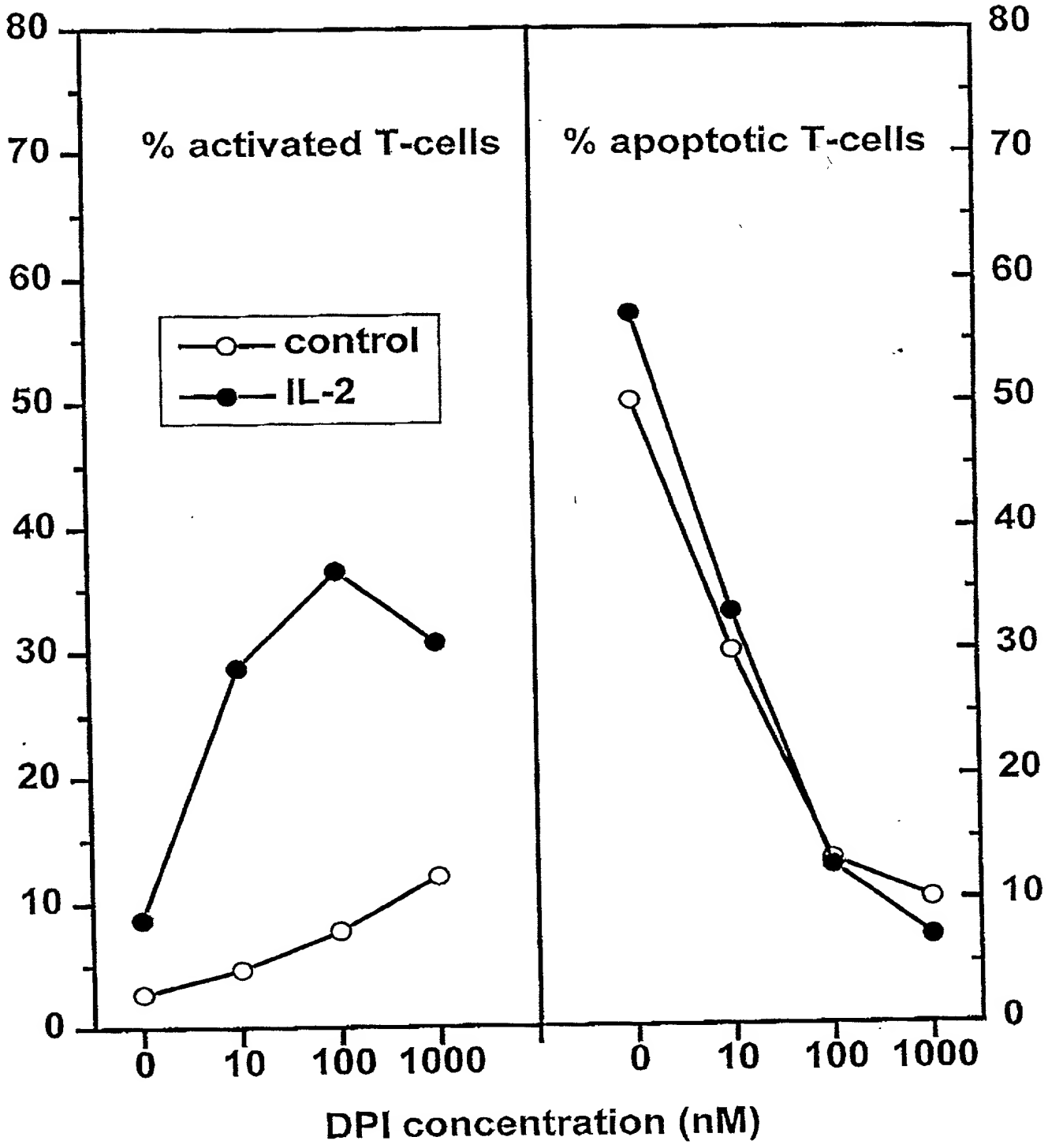
FIGURE 1


% activated T-cells
% apoptotic T-cells
control
IL-2
80
70
60
50
40
30
20
10
0
0
10
100
1000
DPI concentration (nM)